AD________________

Award Number: W81XWH-12-1-0246

TITLE: A functional high-throughput assay of myelination in vitro

PRINCIPAL INVESTIGATOR: Michael J. Moore

CONTRACTING ORGANIZATION: Tulane University
New Orleans, LA 70118

REPORT DATE: July 2013

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. **PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.**

1. REPORT DATE July 2013	**2. REPORT TYPE** Annual	**3. DATES COVERED** 1 July 2012 - 30 June 2013

4. TITLE AND SUBTITLE A functional high-throughput assay of myelination in vitro	**5a. CONTRACT NUMBER**
	5b. GRANT NUMBER W81XWH-12-1-0246
	5c. PROGRAM ELEMENT NUMBER
6. AUTHOR(S) Michael J. Moore	**5d. PROJECT NUMBER**
	5e. TASK NUMBER
E-Mail: mooremj@tulane.edu	**5f. WORK UNIT NUMBER**
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Tulane University New Orleans, LA 70118	**8. PERFORMING ORGANIZATION REPORT NUMBER**
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012	**10. SPONSOR/MONITOR'S ACRONYM(S)**
	11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Effective *in vitro* models of myelination, dysmyelination, and/or remyelination would substantially speed the development and testing of potential therapies for myelin disorders such as multiple sclerosis. Tissues engineered from human induced pluripotent stem (iPS) may be effective at accurately modeling aspects of human physiology for screening of potential therapies. Substantial progress has been made toward demonstrating the feasibility of developing microengineered human neural tissues that can be assessed non-invasively. A population of neurons has been derived from human iPS cell lines, a micropatterned hydrogel culture system has been identified for the support of human neurons, and an optical method for monitoring physiological responses in microengineered tissue constructs has been demonstrated. This works represents a unique combination of enabling technologies, including human iPS cells, microfabrication, and optical neural recording, with the goal of showing how it may be possible to create a high-throughput assay of human neural activity.

15. SUBJECT TERMS
Human induced pluripotent stem cells, hydrogels, 3D culture, electrophysiology, high-throughput assay

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON** USAMRMC
a. REPORT U	**b. ABSTRACT** U	**c. THIS PAGE** U	UU	8	**19b. TELEPHONE NUMBER** *(include area code)*

Table of Contents

Introduction

Effective *in vitro* models of myelination, dysmyelination, and/or remyelination would substantially speed the development and testing of potential therapies for myelin disorders such as multiple sclerosis. Tissues engineered from human induced pluripotent stem (iPS) may be effective at accurately modeling aspects of human physiology for screening of potential therapies. The most physiologically relevant measure of myelin integrity is compound action potential conduction velocity, which is currently not feasible to measure *in vitro* because of a lack of tissue culture models that mimic myelinated neural tracts. The overall objective of this project is to establish the feasibility of noninvasively assessing myelin integrity with an electrophysiological endpoint in biomimetic, organotypic neural tissue cultures engineered from human iPS-derived cells. The aims of this project are to 1) develop an *in vitro* model of myelinated nerve fiber tracts derived from human iPS cells; and 2) to demonstrate the feasibility of noninvasive, optical stimulation and recording from microengineered tissues. All progress and results discussed in this report are in regard to the revised Statement of Work approved as part of the amended contract effective 4 June 2013.

Body

Significant progress has been made toward completion of Task 1. Human iPS cells were obtained from NuPotential, Inc. These cells had been derived from human astrocytes and reprogrammed by lentiviral induction of pluripotent genes Oct4, Sox2, Klf4, and c-MYC. These cells were cultured on feeder layers of confluent, non-mitotic embryonic mouse fibroblasts. Culturing in a commercial neural induction medium (*life* Technologies, Inc.) produced embryoid bodies (EBs), which were passaged at different frequencies to generate eight different cell lines. The phenotype of each of these cell lines was monitored by checking for spindle-like morphology, by RT-PCR for expression of neuronal markers nestin and MAP-2, and by immunocytochemistry for expression of nestin and MAP-2 proteins. Gene expression was quantified relative to a the housekeeping gene GAPDH. As shown in Figure 1, neuronal induction of iPS cells produced EBs, but formation of intermediate neural rosettes was not observed as expected, even though neuronal maturation was evident from morphology and expression of neuronal proteins.

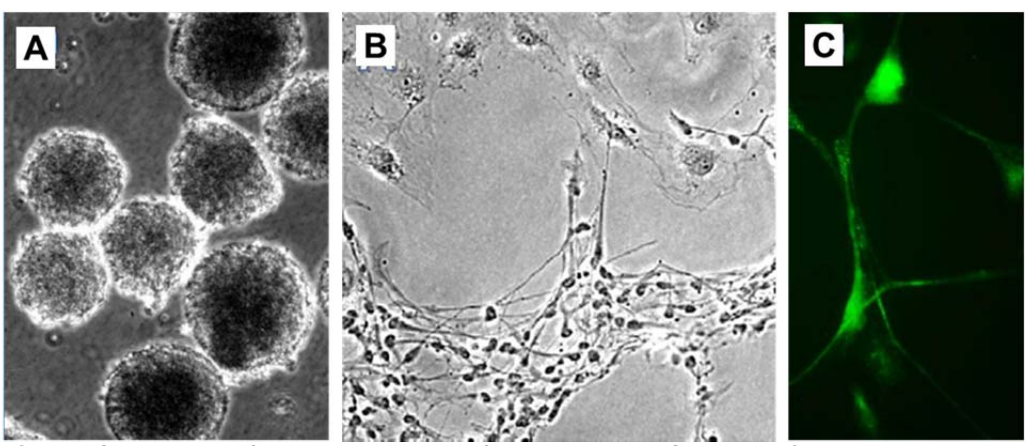

Fig 1: Cultivation of neurons derived from human iPS cells. **A)** Typical embryoid bodies. **B)** Mixed population of cells not showing neuronal morphology (top portion of figure) as well as cells with clear neuronal phenotype (bottom). **C)** Neurons staining positive for MAP-2 protein.

Three of the eight cell lines (lines 1, 2, and 8) showed higher levels of both nestin and MAP-2, and immunocytochemistry indeed confirmed protein expression (Fig. 2). Initial results indicate that substantial neuronal induction has taken place, but colonies will likely need to be further enriched in order to be viable as a reproducible source of neurons for hydrogel cultures. Further characterization of the remaining cell lines, as well as monitoring of glial phenotypes, is being completed presently. We will also attempt to adapt newly described procedures for production of enriched neuronal populations from iPS cells (Zhang et al., Neuron 78:785, 2013). However, changes in personnel have slowed the progress of stem cell culture. In summary, we have successfully cultivated neurons from human iPS cells, as confirmed by morphology, immunostaining, and PCR. However, cell populations remain impure, and so we will take steps to enrich cell populations for a higher proportion of neurons.

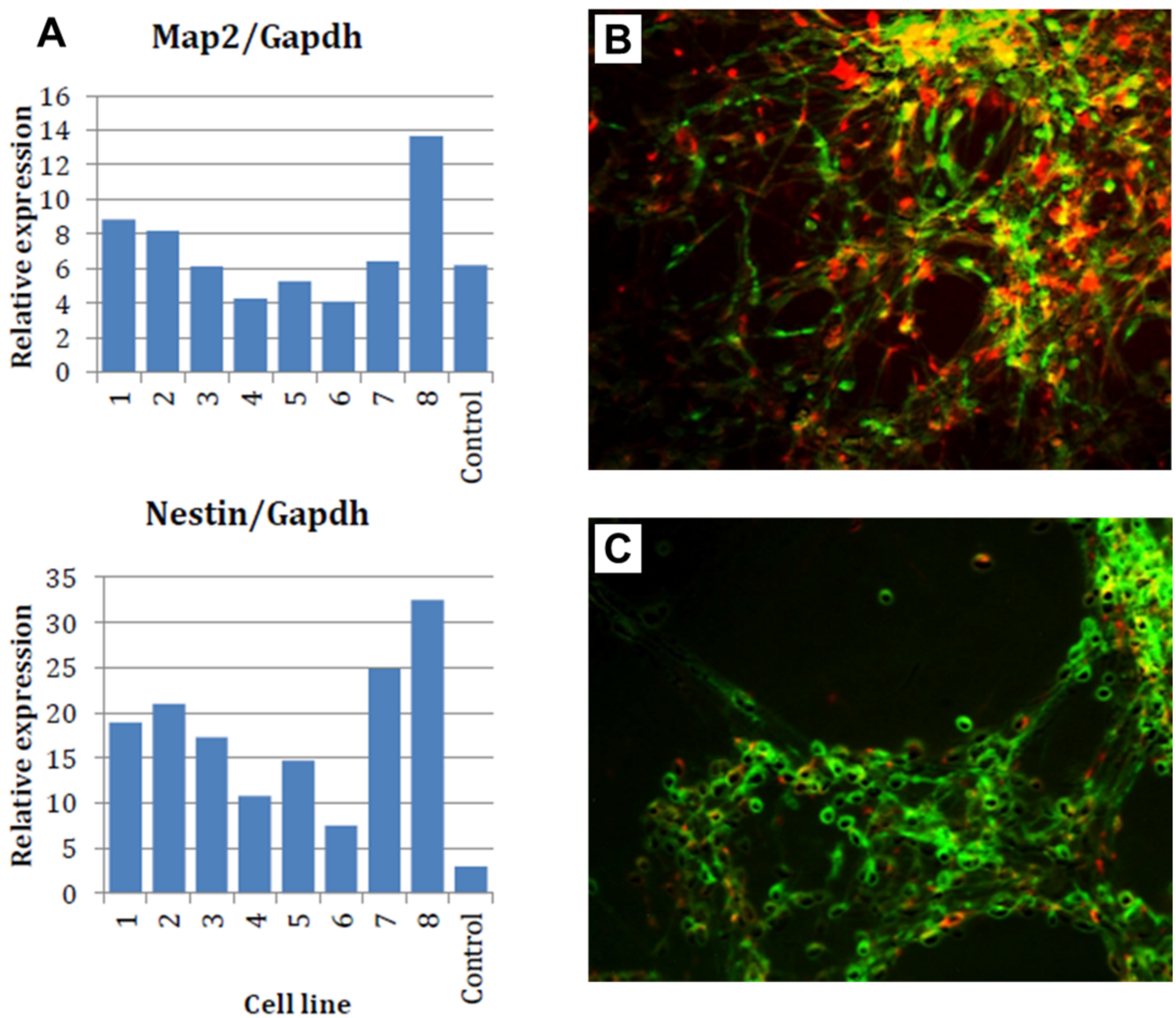

Fig 2: Expression of neuronal markers. **A)** Expression of neuronal genes MAP-2 (top) and nestin (bottom) relative to GAPDH, as compared to undifferentiated control iPS cells. Representative images of nestin (red) and MAP-2 (green), taken from cell line 1, show variable levels of protein expression. High levels of both proteins are depicted in (**B**), while relatively lower levels of nestin are depicted in (**C**).

In regard to Task 2, we have successfully synthesized a photocrosslinkable hyaluronic acid (HA) hydrogel and have been able to incorporate the HA gel into micro-molds of crosslinked polyethylene glycol (PEG). Unfortunately, achieving robust quantities of neurite growth from iPS-derived neurons in hydrogels has proven challenging. As a first step, we cultured non-terminally differentiated neurospheres in both HA gels and Puramatrix gels (which we have used previously with primary embryonic rat neurons) and observed that neurite growth was supported by both gels but was consistently more robust in Puramatrix gels, as demonstrated in Figure 3. For this reason, we have also made an interpenetrating network of crosslinked HA blended with Puramatrix.

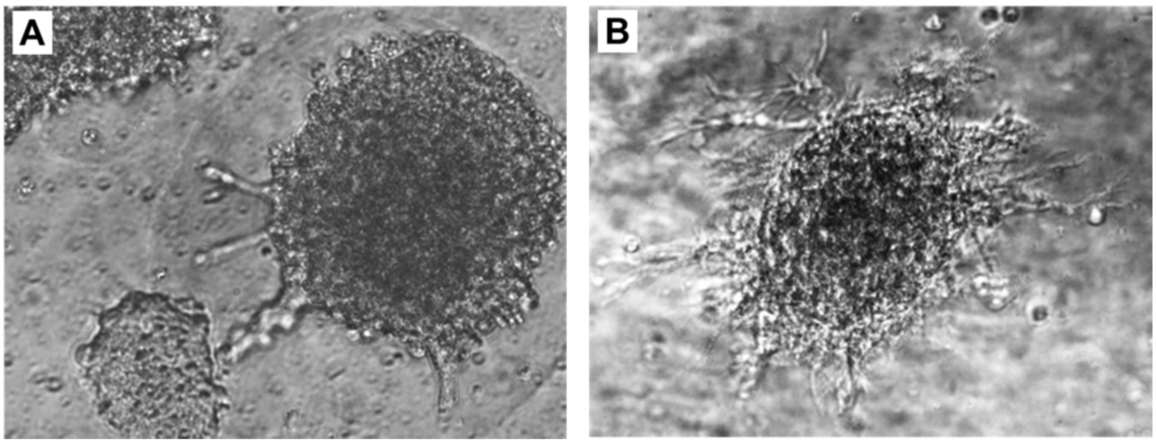

Fig 3: Representative phase-contrast images of neurite growth from neurospheres cultured in HA (**A**) and Puramatrix (**B**) hydrogels.

Further, we have found that neurite growth from whole neurospheres is not adequately robust or reproducible, probably due to impure populations of undifferentiated cells mixed among mature neurons and glia. Therefore, we have determined we should instead start with an enriched population of differentiated neurons to be incorporated into the micropatterned hydrogel system, within which we will then introduce growth factors to promote neurite growth. This effort is ongoing.

Some preliminary progress has been made toward completion of Task 3.1. To demonstrate the feasibility of detecting action potential propagation in 3D hydrogel cultures with voltage-sensitive dye imaging, we have begun to use rat embryonic dorsal root ganglion (DRG) explants suspended in a Puramatrix gel contained within a PEG micro-mold (Curley, 2011; Horn-Ranney, 2013). We have obtained a newly-described voltage-sensitive dye as a generous gift from the lab of Roger Tsien. These new dye, dubbed “molecular wires,” enable fast and direct sensing of membrane voltage changes with larger dynamic ranges than calcium dyes or traditional, electrochromic voltage-sensitive dyes (Miller, 2012). In a preliminary experiment, we simply added the dye at a final concentration of 200 nM to the DRG in the patterned hydrogels. We then forced depolarization of the entire culture by addition of 50 mM KCl to produce a rapid and robust depolarization of all cells in the culture in an effort to ensure detection of a fluorescence signal.

As shown in Figure 4 A and B, when fluorescence change was visualized as a change in fluorescence intensity normalized to pre-KCl intensity ($\Delta I/I_0$), some regions of the ganglion clearly indicated a positive change. The maximum change was then plotted over time, (instead of the mean change over all pixels, which was overwhelmed by a lack of change in the

background fluorescence). As shown in Figure 4 C, there is a clear increase in intensity immediately after KCl addition, suggesting that the dye successfully detected K^+-induced depolarization. Further experiments with electrically- and optically-evoked stimulation need to be carried out in this preparation, followed by experiments with neurons derived from human iPS cells. In summary, we have demonstrated the feasibility of detecting induced action potential propagation in 3D cultures using newly-described voltage-sensitive dyes.

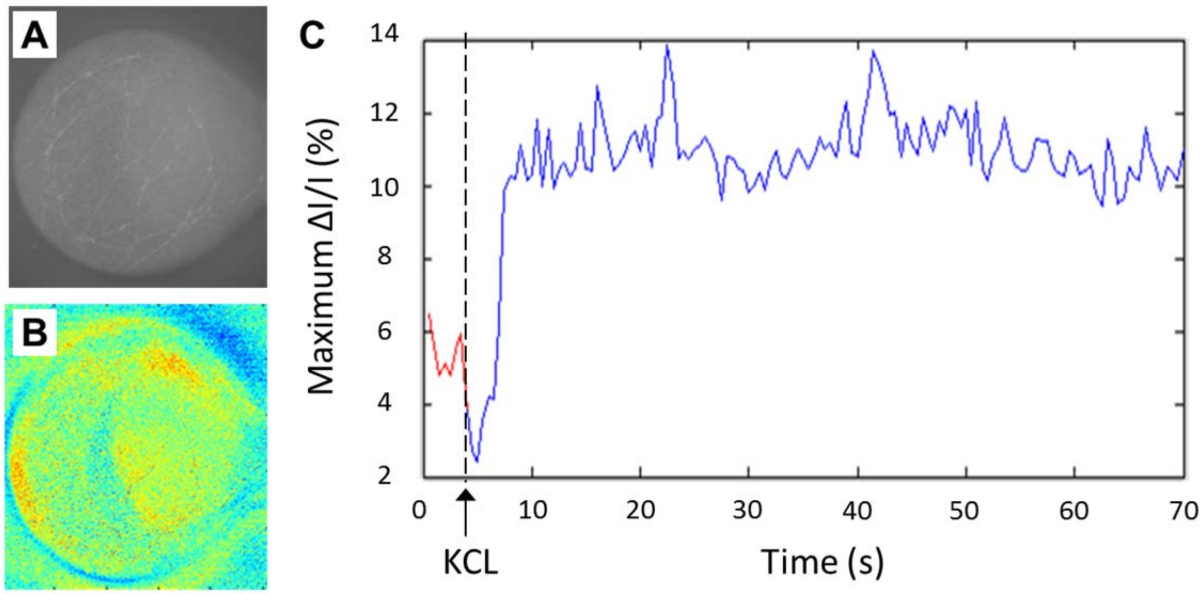

Fig 4: Voltage-sensitive dye imaging of embryonic DRG neurons upon KCl depolarization. **A)** Fluorescence micrograph of DRG explant in a Puramatrix hydrogel inside of a PEG micro-mold. A subset of neurons stained with the dye fluoresce brightly, while background stain is apparent in the remainder of the ganglion, and the PEG mold fluoresces very little. **B)** Visual depiction of change in fluorescence ($\Delta I/I_0$) after forced depolarization with 50 mM KCl. Red regions represent an increase in fluorescence (depolarization) while blue regions indicate a decrease. **C)** Plot of maximum fluorescence change ($\Delta I/I_0$) with time, indicating a depolarization after addition of KCl.

Key Research Accomplishments

- Human iPS cells have been converted to neuronal phenotypes.
- HA hydrogels and interpenetrating networks with Puramatrix have been synthesized and used to support 3D culture of neural stem cells.
- Rat DRG explants cultured in 3D hydrogels have been shown to be amenable to detection of depolarization events with voltage-sensitive dye imaging.

Reportable Outcomes

- At least one cell line of human iPS cells has been generated that has been shown to be capable of producing substantial quantities of neurons.

Conclusions

In summary, substantial progress has been made toward demonstrating the feasibility of developing microengineered human neural tissues that can be assessed non-invasively. A population of neurons has been obtained from human iPS cell lines, but neuronal populations must be further enriched in order to be practical sources of microengineered human tissue. A hydrogel culture system has been identified for the support of human neurons, and an optical method for monitoring evoked electrophysiological responses in microengineered tissue constructs has been shown to be feasible. This works represents a unique combination of enabling technologies, including human iPS cells, microfabrication, and optical neural recording, with the goal of showing how it may be possible to create a high-throughput assay of human neural activity. Such an assay may prove to be useful for modeling neurological disorders, such as multiple sclerosis, and for screening potential therapies.

References

Curley JL, Moore MJ. Facile micropatterning of dual hydrogel systems for 3D models of neurite outgrowth. *J Biomed Mater Res A* 2011, 99(4):532-43.

Horn-Ranney EL, Curley JL, Catig GC, Huval RM, Moore MJ. Structural and molecular micropatterning of dual hydrogel constructs for neural growth models using photochemical strategies. *Biomed Microdevices* 2013, 15(1):49-61.

Miller EW, Lin JY, Frady EP, Steinbach PA, Kristan WB, Tsien RY. Optically monitoring voltage in neurons by photo-induced electron transfer through molecular wires. *Proc Natl Acad Sci USA* 2012, 109(6):2114-9.

Zhang Y, Pak CH, Han Y, Ahlenlus H, Zhang Z, chanda S, Marro S, Patzke C, Acuna C, Covy J, Xu W, Yang N, Danko T, Chen L, Wemig M, Sudhof TC. Rapid single-step induction of functional neurons from human pluripotent stem cells. *Neuron* 2013, 78:785-98.